II. REMARKS

Claims 1 to 20 are pending in the subject application. Claims 1-4, 6-14, and 16-20, are withdrawn from examination as being drawn to non-elected inventions pursuant to 35 U.S.C. § 121. Claims 5 and 15 are currently under examination.

By this Amendment and Response, claim 5 has been amended without prejudice or disclaimer to recite that the FRP-4 protein is a mammalian frizzled related protein 4 ("FRP-4"). The amendment to claim 5 is not intended to be a dedication to the public of the subject matter of the claim as originally filed. Applicants reserve the right to file the same or similar claim in a continuation application.

Support for the amendment to claim 5 is found in the specification on page 9, lines 12 and 13. Accordingly, an issue of new matter is not raised by this amendment and entry thereof is respectfully requested.

The specification also has been amended to remove embedded hyperlinks and/or other forms of browser-executable code. An issue of new matter is not raised by these amendments.

In view of the preceding amendments and remarks that follow, reconsideration and withdrawal of the objections and rejections are respectfully requested.

Objection to the Specification

The specification was objected to because it contains embedded hyperlinks and/or other forms of browser-executable code. The Office noted that attempts to incorporate subject matter into the patent application by reference to a hyperlink and/or other forms of browser-executable code is considered to be an improper incorporation by reference.

In response to the Office's objection, the specification has been amended to remove references to hyperlinks and/or other forms of browser-executable code. In view of these amendments, reconsideration and withdrawal of the objection to the specification is respectfully requested.

35 U.S.C. § 112, Second Paragraph

The specification was objected to, and claims 5 and 15 stand rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite for use of the abbreviation "FRP-4" in claims 5 and 15. In response to the Office's rejection, claims 5 and 15 have been amended to incorporate the name of the protein in its complete form. Support for this amendment is found throughout the specification, for example on page 1, lines 8 and 9; page 5, lines 12-17; and page 7, lines 5-9. In view of this amendment, reconsideration and withdrawal of the objection to the specification and rejection of claims 5 and 15 under 35 U.S.C. § 112, second paragraph, is respectfully requested.

35 U.S.C. § 112, First Paragraph

Claim 5 stands rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Office alleged that claims 5 and 15 read on any FRP-4 protein, including sequences from any species, mutated sequences, polymorphic and allelic variants, splice variants, sequences that have an unspecified degree of identity (similarity, homology), and so forth. The Office opined that the specification as filed provides only a description of human frizzled-related protein, FRP-4, polynucleotide and amino acid sequences (see SEQ ID NOs. 1 and 2 (Figures 1 and 2), respectively), but does not provide sufficient description that would allow one of skill in the art to use SEQ ID Nos. 1 and 2 to predict the structures of any FRP-4 protein isolated from other sources, including all polymorphic, allelic and variants of this protein.

Applicants respectfully traverse. Without conceding correctness of the Office's position, claims 5 and 15 are amended to the use of a mammalian FRP-4 protein. Support for this amendment is found on page 9, lines 12 and 13 and page 15, lines 29 to 31, of the specification. The specification enables the making and using of these proteins because the specification as filed discloses the complete amino acid sequence of human FRP-4 protein and the amino acid

sequences of murine and rodent were publicly available at the time the application was filed from journal articles and in gene and protein databases. Applicants' have attached for the Office's convenience the results of a search on the GenBank database for all proteins and nucleic acids known for FRP-4. As the Office will note, human, murine and rodent sequences were publicly available at the time the application to which the subject application claims the benefit of, was filed.

In support of their position, Applicants note that the law does not require an applicant for a patent to provide in the specification information well known in the art. (See, e.g., In re Buchner, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991)). Thus, Applicants' specification and the publicly available information fully enables the scope of the claims.

Claims 5 and 15 also stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Office alleges that, in view of statements found in the literature and the general unpredictability in the art, that the specification does not provide reasonable expectation that the proteins will perform the object of the claim when administered *in vivo*. The Office concluded that therefore the quantity of experimentation required to practice the invention would include the *de novo* determination of how to engineer and deliver a FRP-4 protein such that phosphate re-absorption would be reduced in a subject, particularly, in view of the obstacles needed to overcome to use FRP-4 protein therapy.

Applicants respectfully traverse and maintain that the scope of the amended claims are fully supported by the specification as filed and information available to the public at the time the application (and the application to which the subject application claims the benefit of) was filed.

The law does not require a specification to provide working examples of all embodiments encompassed by the claims, nor is it required that any one embodiment have been actually reduced to practice for satisfaction of the enablement requirement of 35 U.S.C. § 112, first paragraph. In re Wright, 999 F.2d 1557, 27 U.S.P.Q.2d 1510 (Fed. Cir. 1993); In re Long, 368 F.2d 892, 151 U.S.P.Q. 640 (C.C.P.A. 1960).

Applicants' specification enables one to make and use the invention of the claims, see for example page 9, lines 7-13; page 12, lines 20-22; and page 23, lines 1-11 and 22-32. The specification clearly sets forth how to reduce the invention of the claims to practice, without an undue amount of specification. In support of their position, Applicants attach hereto a copy of their manuscript accepted for publication which discloses data showing that FRP-4 protein infusions specifically increased renal fractional excretion of inorganic phosphate and decreased serum phosphate. Thus, FRP-4 was shown by Applicants to reduce phosphate re-absorption *in vivo*, after administration *in vivo*, consistent with the teachings of the specification as filed.

In view of the preceding amendments, remarks, and attached manuscript, Applicants have overcome the rejections of the claims under 35 U.S.C. § 112, first paragraph and removal of these rejections are respectfully requested.

III. CONCLUSION

No additional fee is deemed necessary in connection with the filing of this Response. However, if the Patent Office determines that an extension and/or other relief is required, Applicants petition for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 50-2518**, referencing billing number **7009072002**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Should a telephone advance prosecution of the subject application, the Examiner is invited to contact the undersigned at (650) 849-4950.

Respectfully submitted,

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Secreted Frizzled-Related Protein 4 is a Potent Tumor-derived Phosphaturic Agent

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Abstract:

Tumors associated with osteomalacia elaborate a novel factor(s), "phosphatonin(s)", which causes phosphaturia and hypophosphatemia by cAMP-independent pathways. We show that secreted frizzled-related protein-4 (sFRP-4), a protein highly expressed in such tumors, is a circulating phosphaturic factor that antagonizes renal Wnt-signaling. In cultured opossum renal epithelial cells, sFRP-4 specifically inhibited sodium-dependent phosphate transport. sFRP-4 infusions in normal rats over 2h specifically increased renal fractional excretion of inorganic phosphate (FE_{Pi}) from 14±2% to 34±5% (mean±SEM, $p<0.01$). Urinary cAMP and calcium excretion were unchanged. In thyro-parathyroidectomized rats, sFRP-4 increased FE_{Pi} from 0.7±0.2% to 3.8±1.2% ($p<0.05$), demonstrating that sFRP-4 inhibits renal inorganic phosphate reabsorption by parathyroid hormone-independent mechanisms. Administration of sFRP-4 to intact rats over 8h increased FE_{Pi}, decreased serum phosphate (1.95±0.1 to 1.53±0.09 mmol/L, $p<0.05$) but did not alter serum 1α, 25-dihydroxyvitamin D, renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 and sodium-phosphate co-transporter mRNA concentrations. Infusion of sFRP-4 antagonizes Wnt action as demonstrated by reduced renal β-catenin and increased phosphorylated β-catenin concentrations. sFRP-4 is detectable in normal human serum and in the serum of a patient with tumor-induced osteomalacia. Thus, sFRP-4 displays "phosphatonin" like properties, as it is a circulating protein that promotes phosphaturia and hypophosphatemia, and blunts compensatory increases in 1α, 25-dihydroxyvitamin D.

Key words

Secreted frizzled-related protein-4 (sFRP-4), phosphate, cAMP (cyclic adenosine monophosphate), 1α, 25-dihydroxyvitamin D_3, parathyroid hormone, fractional excretion of phosphate, Wnt.

Introduction:

Tumor induced osteomalacia (TIO) is a rare syndrome associated with hypophosphatemia, excessive renal phosphate excretion, osteomalacia and abnormal vitamin D metabolism (1-8). Tumors associated with this syndrome are usually of mesenchymal origin and are believed to elaborate a circulating factor known as "phosphatonin", which is responsible for the syndrome (1-8). Complete removal of such tumors is associated with remission of the biochemical and skeletal abnormalities. In contrast to hyperparathyroidism and humoral hypercalcemia of malignancy, serum calcium, parathyroid hormone (PTH) and parathyroid hormone related protein (PTHrP) concentrations are generally normal in TIO (2-5). Serum 1α, 25-dihydroxyvitamin D concentrations, which would be expected to be increased in the presence of hypophosphatemia, are normal or reduced (2-5).

Previously, we showed that a tumor associated with this syndrome secreted a factor (or factors) that had biological properties distinct from those of other known phosphaturic proteins such as PTH and PTHrP (2). Like PTH and PTHrP, tumor supernatants inhibited sodium-dependent phosphate transport, but not sodium-dependent glucose or amino acid transport, in cultured opossum kidney (OK) cells. In contrast to the actions of PTH and PTHrP, which are mediated by 3', 5' cyclic adenosine monophosphate (cAMP), tumor cell supernatants inhibited sodium-dependent phosphate transport without altering cAMP concentrations. The inhibitory effect of tumor supernatants on sodium-dependent phosphate transport was not blocked following treatment with a PTH receptor antagonist

further indicating that the substance present in tumor supernatants was not PTH or PTHrP. This factor was named "phosphatonin" (9) to distinguish it from other known phosphaturic proteins. These findings have been subsequently confirmed by other investigators (10, 11).

Until recently, the chemical identity of "phosphatonin" has been elusive. Work by several groups demonstrated that fibroblast growth factor 23 (FGF23) is expressed in tumors associated with TIO (12-15). We, and subsequently, others demonstrated that FGF-23 specifically inhibited phosphate transport *in vitro* (12, 16) . Furthermore, FGF-23 administration or over-expression in animals reproduces the renal phosphate wasting, and osteomalacia observed in TIO patients (16-18). The recent demonstration that some patients with TIO have elevated serum FGF-23 levels (19, 20) further supports the hypothesis that FGF-23 is a "phosphatonin".

Coincident with the above studies, we performed serial analysis of gene expression (SAGE) of four tumors associated with renal phosphate wasting to identify the most highly and differentially expressed genes present in such tumors (21). In addition to *FGF-23*, we identified several other genes, including *secreted frizzled-related protein 4* (*sFRP-4*), as "phosphatonin" candidates. Given that sFRP-4 is a highly and differentially expressed, secreted protein with a previously unknown function, we investigated its potential as a "phosphatonin".

To test whether sFRP-4 inhibits renal sodium-dependent phosphate transport, we monitored the effect of recombinant sFRP-4 on radiolabeled inorganic phosphorus uptake in renal epithelial cells *in vitro,* and we intravenously infused recombinant sFRP-4 in rats and mice to assess its affect on solute transport *in vivo.* We now report that sFRP-4 is a potent phosphate-regulating agent *in vitro* and *in vivo*, and that it acts independently of PTH. Furthermore, sFRP-4 disrupts the compensatory increase in renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 messenger RNA concentrations and serum 1α, 25-dihydroxyvitamin D concentrations typically observed in response to hypophosphatemia. To determine whether sFRP-4 is indeed a circulating factor as is characteristic of "phosphatonin", we measured and detected sFRP-4 in normal human serum and in the serum of a patient with TIO. Moreover, we demonstrate that sFRP-4 functions as an antagonist of Wnt-signaling in the kidney as the administration of sFRP-4 *in vivo* is associated with a decrease in total β-catenin and an increase in phosphorylated β-catenin in renal tissues. Our data show that sFRP-4 could potentially function as a "phosphatonin". Thus, tumors associated with osteomalacia elaborate at least two phosphaturic factors, FGF-23 and sFRP-4.

Methods and Materials:

Synthesis and Purification of Secreted Frizzled-Related Protein-4: A cDNA pool was synthesized from a TIO tumor using the Lambda ZAP-CMV cDNA synthesis kit (Stratagene, La Jolla, CA) (22, 23). Full-length human *sFRP-4* cDNA containing the open reading frame minus the stop codon was amplified from the cDNA pool using the sense primer 5'GCAGTGCCATGTTCCTCTCCATCC3' and the antisense primer 5'CACTCTTTTCGGGTTTGTTCTC3' and high fidelity *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) (22, 23). The amplicon was cloned in frame with the V5-His epitopes into pcDNA3.1-V5-His/TOPO (Invitrogen Corp., Carlsbad, CA) or pIB/V5-His insect vector (Invitrogen Corp., Carlsbad, CA) (22, 23) and sequence fidelity confirmed (Sequegen, Worcester, MA). BTI-TN-5B1-4 (High Five™) *Trichoplusia ni* insect cells were stably transformed with pIB/V5-His-sFRP-4-V5his6X and grown in Express Five serum-free medium supplemented with 90 ml of 200 mM L-Glutamine/liter (Invitrogen Corp.) and blasticidin S for selection and maintenance of stock cultures, in 75 cm^2 bottles (InvivoGen, San Diego, CA). For large-scale expression of sFRP-4, a 2-L Ehrlenmeyer flask containing 0.5 L Express Five medium was inoculated with ~0.5-1 x 10^6 cells/ml and grown at 27^0 C and 120 rpm in an Innova 4330 temperature-controlled incubator-shaker (New Brunswick Scientific Co., Edison, NJ). Conditioned media was obtained after 5-6 days of culture growth (final cell density ~0.4-1 x 10^7 cells/ml). Secreted FRP-4 was adsorbed from filtered conditioned medium to 25 ml SP Sepharose resin (Amersham Biosciences, Piscataway, NJ) for ~12 hr at 4^0 C with stirring. The resin was batch washed sequentially, four times with each buffer using 10 volumes of 20 mM Na_2HPO_4 (pH 7.4), 65 mM NaCl (start buffer), followed by 20 mM Na_2HPO_4 (pH 7.4),

200 mM NaCl (intermediate buffer). Secreted FRP-4 was eluted with three washes of 50 ml of 20 mM Na_2HPO_4 (pH 7.4), 1 M NaCl. The eluate was dialyzed into 500 ml start buffer at 4 ^{0}C using Spectra/Por 7 dialysis tubing (1000 molecular weight cut-off, Spectrum Medical Industries, Laguna Hills, CA) with 3 changes of buffer. The dialysate was filtered (0.2 μm, 25 mm nylon syringe filter, Nalge Co., Rochester, NY) and applied to a Hi Prep™16/10 FF SP Sepharose column using an ÄKTA FPLC protein purification system (Amersham Biosciences). Secreted FRP-4 protein was eluted using a programmed elution gradient starting with 20 mM Na_2HPO_4 (pH 7.4), 65 mM NaCl and ending with 20 mM Na_2HPO_4 (pH 7.4), 1.0 M NaCl. Fractions containing sFRP-4 were identified by anti-V5 immunoblot analysis, concentrated using Centriprep 10 units (Amicon, Beverly, MA), and sterile filtered using 0.2 μm nylon syringe filters. Secreted FRP-4 protein had the expected N-terminal amino acid sequence (APC/XEAV).

Effect of sFRP-4 on phosphate transport in OK cells. Opossum-kidney cells were cultured in 45% Dulbecco's modified Eagle's medium, 45% F12 medium, with 10% fetal-calf serum. The cells were seeded at a density of about 5 x 10^3 per well in 24-well tissue-culture plates and sodium-dependent phosphate, alanine, and glucose co-transport measured as described previously (2, 12). In brief, sFRP-4 or vehicle was added in varying amounts to growth medium to attain concentrations shown in Figure 1. For the measurement of sodium-dependent phosphate transport, 0.1 mM dibasic potassium phosphate was included in the transport medium and ^{32}P dibasic potassium phosphate was added to a final specific activity of 2 μCi/ml. For sodium-dependent alanine transport, 0.1 mM l-alanine and ^3H-alanine were added (final specific activity, 1 μCi/ml). For

glucose transport, 0.1 mM methyl-α-glucopyranoside and methyl (α-d- [^{14}C] gluco) pyranoside were added (final specific activity, 0.2 μCi/ml). The transport of phosphate, alanine, and methyl-α-glucopyranoside were assayed separately. Each transport reaction was measured in three or four duplicate wells and each assay included control blank wells to correct for solute bound to cell surfaces, intracellular spaces, and culture dish.

Animals: All animal protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Male Sprague Dawley rats weighing 250-300 grams were purchased from Harlan Sprague Dawley (Madison, Wisconsin). They were fed a standard rodent formula containing 0.7% phosphorus and 0.5% calcium and adequate amounts of vitamin D. On the day of the acute experiments, rats were anesthetized with an intra-peritoneal injection of 100-150 mg/kg body wt of 5-sec-butyl-ethyl-2-thiobarbituric acid (Inactin: Byk-Gulden Konstanz, Hamburg, Germany). The animals were placed on a heated table to maintain body temperature between 36 and 38°C. After a tracheostomy, a PE-50 catheter was placed in the left carotid artery to monitor mean arterial blood pressure (MAP) and to collect blood samples. Another catheter was placed in the left jugular vein for intravenous infusion of 1% inulin in 0.9% NaCl and 2.25% BSA at a rate of 1% BW per hour and for drug administration. A PE 90 catheter was placed in the bladder for urine collection. The groups of animals studied were as follows:

Short-term (2 hour) infusion of vehicle (group 1, n = 7) or sFRP-4 (group 2, n = 10). Effects on solute excretion in normal rats. After a 1.5-h recovery period, one 30-min urine clearance sample was taken (C1). Then, approximately 35 μl of vehicle that

contained PBS and 0.1% BSA (Group 1) or sFRP-4 in vehicle (Group 2, 0.3 μg/kg/hr of sFRP-4), was added to the inulin/BSA infusion. After a 45-min stabilization period, a 60-minute urine clearance (C2) was taken from either vehicle-treated or sFRP-4 treated animals. A blood sample was taken at the midpoint of the clearance period. At the end of the experiment, the kidneys were removed for isolation of total RNA and quantitation of 25-hydroxyvitamin D 1α-hydroxylase and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNAs.

Short-term (2-hour) infusion of vehicle (group 3, n = 6) or sFRP-4 (group 4, n = 10). Effect on solute excretion in thyro-parathyroidectomized (TPTX) rats. These protocols are identical to those described for groups 1 and 2, except that TPTX rats were studied two hours after the completion of thyro-parathyroidectomy. Effective thyro-parathyroidectomy was confirmed by a significant reduction in the urinary fractional excretion of phosphate, increased fractional excretion of calcium and the presence of hypocalcemia.

Long-term (8-hour) infusion of vehicle (group 5, n = 5) or sFRP-4 (group 6, n = 7). Effects on solute excretion, serum 1,25-dihydroxyvitamin D concentrations and 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 and *25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNA concentrations.* These protocols are identical to those described for the previous groups, except that after the control collection (C1), one hour clearances were taken at 4 hours (C2) and 7 hours (C3) after initiation of the vehicle or sFRP-4 infusions. At the end of the experiment, blood was collected for measurement

of 1α, 25-dihydroxyvitamin D, and kidneys were collected for the isolation of mRNA and assessment of 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNA concentrations by quantitative real-time RT-PCR. To determine if decrements in serum phosphate were associated with appropriate increases in renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 mRNA concentrations, rats were maintained on a 0.1% phosphate diet (low Pi) or a 0.4% phosphate diet (normal Pi) for a period of one week, so as to bring about a modest reduction in serum phosphate equivalent to that seen following sFRP-4 infusion.

Serum and urine measurements: Serum and urinary phosphate concentrations were determined using the method of Chen *et al.* (24). Serum and urine inulin concentrations were measured using the anthrone method (25). Sodium concentrations were measured in urine using an Instrumentation Laboratory flame photometer (Instrumentation Laboratory, Wilmington, MA). Serum and urine calcium concentrations were determined by atomic absorption spectrometry (26-29). Plasma 1α, 25-dihydroxyvitamin D and 25-hydroxyvitamin D concentrations were measured by radio-immunoassay (Diasorin, Stillwater, MN). Urinary cyclic adenosine monophosphate was measured using an enzyme immunoassay (Amersham Biosciences).

Real-time PCR to assess 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 Messenger RNA Concentrations in the Kidney: A Polytron (Brinkman Instruments, Westbury, NY) was used to homogenize

a lateral third of each rat kidney containing cortical tissue in 4 ml of RLT buffer (Qiagen, Valencia, CA). The homogenate was centrifuged for 3 min at 4500 x g. Total RNA was extracted from the supernatant following treatment with DNase using the RNeasy midi kit (Qiagen). RNA concentration in the samples was quantitated using UV spectroscopy at a wavelength of 260 nm. Samples containing 200 ng total RNA were prepared using the Quantitect SYBR Green RT-PCR kit (Qiagen) and real-time PCR done following the manufacturer's protocol on a PE Applied Biosystems Model 7700 sequence detector (Applied Biosystems, Foster City, CA). Primers were designed to span introns and to generate amplicons of 100-120 bp. The primer sequences are as follows: 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 (forward, 5'GCAT CCATCTCCAGTTTGTAGAC3'; reverse, 5'TGTGCCTCTTGTGCATAGTAAGA3'), 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 (forward, 5'GATCACCTTTCCAAGAAGGAACT3'; reverse, 5'AGAGAATCCACATCAAGCTG TTC3'), sodium dependent phosphate co-transporter IIa forward, 5' CTGTGCACTTG TCTTATCCTCCT; reverse 5'GGAAGTCTGTGTTGATGACCTT. Long oligonucleotide templates were designed which represented the entire amplicon sequence for each gene and dilutions of which were used to generate standard curves. All samples were interrogated in triplicate, and the averages of each normalized to GAPDH. GAPDH mRNA values did not deviate significantly across samples. Normalized relative values were assigned absolute values based on the slope and y-intercept of the relevant standard curve. All PCR products, including reverse transcriptase negative control, and no template control samples were subjected to electrophoresis on a 4% agarose gel. Only expected band sizes were detected.

Enzyme-linked Immunosorbent Assay (ELISA) for sFRP-4 in Serum:

A two-antibody sandwich ELISA was developed using monoclonal antibodies (Mab 3.1 and 3.4) against insect-derived sFRP-4 protein. These antibodies were prepared in the Mayo Clinic Core Facility using established procedures (30). Briefly, Mab 3.4 was immobilized onto a Reacti-Bind ELISA plate (Pierce, Rockford, Ill). Recombinant sFRP-4 standards in 100 µl of PBS or 100 µl human serum samples were added to individual wells and incubated at room temperature for 1 hour. Samples were aspirated and wells washed three times with PBS and 0.5% Tween-20. A second anti-sFRP-4 monoclonal antibody, Mab 3.1 was biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, Ill) and applied to each well and incubated for 1 hour at room temperature. Samples were aspirated and wells washed three times with PBS and 0.5% Tween-20. Streptavidin-HRP (100 ng/ml) (Pierce, Rockford, Ill) was added to facilitate detection with 3,3,5,5 tetramethylbenzidine substrate (Sigma, St. Louis, MO). After the addition of stop solution (4ADI, San Antonio, TX), the plate was read in SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) at 450 nm. The concentration of sFRP-4 in each serum sample was calculated based on a standard curve generated using known concentrations of purified insect sFRP-4.

Western blotting to detect β-catenin and phosphorylated β-catenin or sFRP-4 in renal tissues: Kidneys (~ 1 g tissue) from rats infused with sFRP-4 or vehicle, as described, above were homogenized in an equal volume of buffer (50 mM tris (pH 7.8), 150 mM sodium chloride) containing a mammalian protease inhibitor mixture (Sigma Chemical

Co., catalog # P8340) and phosphatase inhibitors, okadaic acid (1 μM) and microcystin LR (1 μM; Sigma Chemicals, St. Louis, MO). Equal amounts of protein from control and experimental kidneys were loaded and electrophoresed on SDS-polyacrylamide gels (31). The electrophoresed proteins were transferred to polyvinylidene difluoride membranes (32), and the membranes were probed with antibodies against β-catenin or phosphorylated β-catenin (Cell Signaling Technologies, Beverly, MA). Horseradish peroxidase conjugated secondary antibodies were used to detect primary antibodies bound to either β-catenin or phosphorylated β-catenin.

For detection of sFRP-4 in renal tissues, rat renal tissue homogenates were electrophoresed on SDS-polyacrylamide gels, and the electrophoresed proteins were transferred to PVDF membranes as described above (31, 32). Monoclonal antibodies raised against sFRP-4 were used to probe PVDF membranes, and bound anti-sFRP-4 antibodies were detected using an anti-mouse IgG, horseradish peroxidase-coupled antibody.

Statistics. Values are expressed as means ± SE. Statistical comparisons for groups 1-4 were made using a paired T-test. Comparisons between multiple clearances (Groups 5 and 6) were made using one-way ANOVA followed by the Fishers PSLD test. Statistical comparison for the phosphate uptake assay was made using an unpaired t-test. A P value <0.05 was considered to be significant.

Results:

Secreted FRP-4 specifically inhibits sodium-dependent phosphate transport in opossum kidney cells: To determine whether sFRP-4 directly inhibits sodium-dependent phosphate transport in opossum kidney cells maintained in culture, we added increasing amounts of sFRP-4 to the supernatants of OK cells maintained in culture (Figure 1). As can be seen, a dose of 250 pg/ml of sFRP-4 per well caused a statistically significant decrease in sodium dependent phosphate uptake in OK cells maintained in culture. Sodium-dependent glucose transport (sFRP-4, 10.26 ± 0.33 nmol/mg protein vs. vehicle, 9.62 ± 0.45 nmol/mg protein, p = NS) and sodium-dependent alanine transport (sFRP-4, 0.059 ± 0.008 nmol/mg protein vs. vehicle, 0.083 ± 0.009 nmol/mg protein. P = NS) did not change significantly.

Effects of short-term sFRP-4 infusion on renal solute transport, renal 25-hydroxyvitamin D 1α-hydroxylase mRNA and 25-hydroxyvitamin D 24-hydroxylase mRNA: As shown in Figure 2, sFRP-4 infused in normal rats over a period of 2 hours caused a marked increase in the fractional excretion of phosphate (FE_{Pi}) from 14 ± 2%, mean ± SEM to 34 ± 5%, mean ± SEM, $p<0.05$. FE_{Pi} was stable (14 ± 3% to 18 ± 2%, mean ± SEM, p = NS) in vehicle infused rats. Fractional sodium excretion (FE_{Na}) increased in the sFRP-4 infused rats from 1.1 ± 0.3% to 2.7 ± 0.5% (mean ± SEM, $p<0.05$). In the vehicle infused group, a smaller increase in FE_{Na} was noted (0.3 ± 0.1% to 1.2 ± 0.3%, mean ± SEM, $p<0.05$). Fractional excretion of calcium (FE_{Ca}) did not change in either group. GFR and mean arterial pressure were stable in both groups of animals (data not shown).

Of note, urinary cAMP excretion did not change in either the sFRP-4-infused (23.6 ± 2.6 to 25.5 ± 2.6 nmol/min, p = NS) or vehicle-infused (24.6 ± 1.2 to 28.2 ± 3.5 nmol/min, p = NS) groups of rats. Serum phosphate and serum calcium concentrations did not change during the short-term infusion in the sFRP-4. Renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 mRNA (sFRP-4: 2.29 x 10^{-13} ± 1.18 x 10^{-13} mol/pg RNA vs. vehicle: 4.36 x 10^{-13} ± 1.82 x 10^{-13} mol/pg RNA, p = NS) and 24-hydroxylase cytochrome P450 mRNA (sFRP-4: 3.73 x 10^{-14} ± 1.35 x 10^{-14} vs. vehicle: 1.45 x 10^{-14} ± 4.0 x 10^{-15} mol/pg RNA, p = NS) concentrations were similar in the sFRP-4 and vehicle treated groups.

Parallel experiments in mice were also performed using recombinant protein derived from mammalian or insect cells. Secreted FRP-4 also induced a 3.1-fold increase in the fractional excretion of phosphate in mice ($p<0.05$). These results confirm the actions of sFRP-4 in another species and demonstrate that the method of production of recombinant sFRP-4 (mammalian vs. insect expression systems) has no major influence on protein function.

The phosphaturic action of sFRP-4 is PTH-independent: To determine whether parathyroid hormone is necessary for the phosphaturic action of sFRP-4, we infused sFRP-4 intravenously in acutely thyro-parathyroidectomized rats. Thyro-parathyroidectomy was associated with a decrease in the basal FE_{Pi} from approximately 15% in intact animals to approximately 0.7% in thyro-parathyroidectomized animals. Basal FE_{Ca} increased from 0.5% to 1.5% and serum calcium decreased from

approximately 10.5 mg/dl to 8 mg/dl. As shown in Figure 3, sFRP-4 infusion in acutely TPTX animals was associated with a 3.5-fold increase in the FE_{Pi} (1.0 ± 0.3% to 3.8 ± 1.2%, $p<0.05$). This 3.5-fold increase during sFRP-4 infusion was similar to that observed in intact animals. The FE_{Pi} was stable in animals receiving the vehicle. There were no changes in the fractional excretion of sodium or calcium in rats infused with sFRP-4. GFR and mean arterial pressure were stable throughout the experiment.

Long-term sFRP-4 infusion results in increased fractional excretion of phosphorus, reduced serum phosphorus concentrations, and inappropriately normal serum 1α, 25-dihydroxyvitamin D concentrations: We examined the effects of a long-term (8 hr) infusion of sFRP-4 on fractional excretion of solutes, serum inorganic phosphorus, serum 1,25-dihydroxyvitamin D concentrations and renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 and 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 messenger RNA concentrations. With long-term infusion of sFRP-4 (Table 1), FE_{Pi} increased from 8.5 ± 2.6% to 20.2 ± 3.7% four hours after initiation of sFRP-4 infusion and was 18.2 ± 4.3% at 8 hours. FE_{Pi} did not change in the animals infused with vehicle. There were no changes in the fractional excretion of sodium or the fractional excretion of calcium in either the sFRP-4 or vehicle groups. In the sFRP-4 infused animals, there was a decrease in serum phosphorus concentrations from 1.95 ± 1.0 mmol/l to 1.51 ± 0.11 mmol/l at 4 h and to 1.53 ± 0.09 mmol/l at 8 h. There was no change in the serum phosphorus concentrations in the animals receiving vehicle. Serum 1α, 25-dihydroxyvitamin D concentrations were not different between the sFRP-4 and vehicle infused rats (89.6 ± 26.8 pg/ml, sFRP-4 group vs. 62.4 ± 29.2 pg/ml vehicle group, mean ± SEM, p = NS).

25-Hydroxyvitamin D 1 α -hydroxylase cytochrome P450 messenger mRNA concentrations were similar in the vehicle and sFRP-4 infused groups at 8 hrs ($1.41x10^{-13}$ ± $3.87x10^{-14}$ moles/pg RNA vs. $1.37x10^{-13}$ ± $1.77x10^{-14}$ moles/pg RNA, p=NS, Figure 4). 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 mRNA concentrations showed a tendency to increase in sFRP-4 infused rats, but this value was not statistically significant. In phosphate-deprived rats, renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 messenger mRNA concentrations were appropriately increased by approximately 3.7-fold ($8.5x10^{-13}$ ± $9.5x10^{-14}$ moles/pg RNA in phosphate-deprived rats, n = 6, vs. $2.3x10^{-13}$ ± $8.9x10^{-14}$ moles/pg RNA in normal rats, n = 6, p <0.05, Figure 4) following an ~16% decrease in serum Pi and an ~33% decrease in urinary phosphate excretion.

The phosphaturic action of sFRP-4 is not mediated through transcriptional regulation of the sodium-dependent phosphate co-transporter: We measured sodium-dependent phosphate co-transporter mRNA concentrations in sFRP-4 treated rats and found that they did not change significantly with sFRP-4 treatment (sFRP-4, $6.93x10^{-11}$ ± $6.41x10^{-12}$ moles/pg RNA vs. vehicle, $8.67x10^{-11}$ ± $9.20x10^{-12}$ moles/pg RNA).

sFRP-4 is detectable in normal human serum and in the serum of a patient with TIO: We measured sFRP-4 concentrations in normal human serum obtained from five normal subjects. The mean concentration was 34.8 ± 13.3 ng/ml and the range was 5.5 to 79.8 ng/ml). In a patient with TIO on with phosphate and 1α, 25-dihydroxyvitamin D_3 serum sFRP-4 was 11.1 ng/ml.

sFRP-4 is expressed in the rat kidneys and antagonizes Wnt-signaling: Immunoblot analysis of rat renal homogenates using a monoclonal anti-FRP-4 antibody indicates that sFRP-4 protein (M_r ~50,000) is detectable in kidney homogenates (data not shown). To determine the mechanism of action of sFRP-4 in the kidney, we measured β-catenin or phosphorylated β-catenin concentrations in sFRP-4 or vehicle treated kidneys *in vivo*. As shown in figure 5, rats infused with FRP-4 have reduced amounts of renal β-catenin protein (upper panel) and increased phosphorylated β-catenin signal (lower panel) compared to those infused with vehicle.

Discussion

Tumor-induced osteomalacia (TIO) is a paraneoplastic renal phosphate wasting disorder characterized by hypophosphatemia, phosphaturia, and inappropriately normal or low serum 1α, 25-dihydroxyvitamin D concentrations that lead to osteomalacia or rickets, muscle weakness and general debility (1-8). In TIO, serum calcium, PTH and PTHrP concentrations are generally normal. The mesenchymal tumors that cause TIO are thought to elaborate a circulating factor that causes the metabolic abnormalities noted above (1-8). Complete removal of the tumors is associated with correction of the biochemical and skeletal abnormalities.

Earlier, we demonstrated that a tumor associated with this syndrome elaborated a novel factor(s) that was responsible for the reduced renal reabsorption of phosphate (2). Recent work suggests that fibroblast growth factor 23 (FGF-23) is a "phosphatonin"-like molecule. In addition to its expression in tumors associated with osteomalacia (13, 15, 33), FGF-23 serum levels are increased in some patients with TIO and X-linked hypophosphatemic rickets (XLH) (19), a syndrome that has phenotypic features similar to TIO (34). Elevated FGF-23 concentrations in the serum of patients with TIO diminish after removal of the tumor and a return of the clinical state to normal (19). FGF-23 reduces phosphate uptake in opossum kidney cells in culture (12, 35) and may be a substrate for the enzyme PHEX (12) that is mutated in patients with X-linked hypophosphatemic rickets (36). Shimada *et al.* showed that recombinant FGF-23 reduces serum phosphate concentrations and increases urinary phosphate losses when administered to mice (13). Furthermore, nude mice implanted with an established cell

line expressing FGF-23 develop osteomalacia. Transgenic mice over-expressing FGF-23 have hypophosphatemia and increased urinary phosphate excretion (37) and null-mutant mice lacking the *FGF-23* gene are hyperphosphatemic (38). Finally, activating mutations of FGF-23 are responsible for autosomal dominant hypophosphatemic rickets (ADHR) (17, 39, 40), which also has clinical features similar to TIO and XLH. ADHR mutations have been associated with increased FGF-23 protein stability and/or activity (16-18, 40).

Our approach to identify potential "phosphatonin" candidates was to perform a comprehensive gene expression profile in four tumors associated with phosphate wasting and to compare these profiles to those of similar mesenchymal tumors not associated with TIO. We examined several tumors associated with the syndrome of TIO for the presence of differentially expressed genes using the technique of serial analysis of gene expression (SAGE) (21, 41, 42). We identified several genes that were expressed preferentially in tumors associated with osteomalacia but not in control tissues. These included *FGF-23, MEPE, dentin matrix protein 1*, and secreted *FRP-4*. Since sFRP-4 is a secreted molecule, we hypothesized that this protein could enter the circulation and reduce phosphate reabsorption in the kidney. To test this hypothesis, we biosynthesized sFRP-4 and demonstrated it specifically inhibited sodium-dependent phosphate uptake in cultured opossum renal epithelia (Figure 1). We further showed that, the infusion of sFRP-4 into rats is associated with a 2.5-3-fold increase in the urinary excretion of phosphate (Figure 2, Table 1). In acute experiments, there is an increase in sodium excretion, which is probably secondary to the infusion itself and is independent of sFRP-4 since it is not apparent in animals infused with sFRP-4 for a period of 8 hours. Urinary fractional

excretion of calcium and urinary cAMP excretion do not change. The infusion of sFRP-4 for a period of 4-8 hours was associated with a decrease in serum phosphate concentrations. Thus, this protein is capable of causing hyperphosphaturia and hypophosphatemia *in vivo*.

To determine whether the phosphaturic effects of sFRP-4 are PTH-dependent, we infused sFRP-4 into acutely thyro-parathyroidectomized rats, and showed that there was a 3-fold increase in phosphate excretion (Figure 3). This is similar to the change in FE_{Pi} observed in the intact animal suggesting that sFRP-4 does not act via parathyroid hormone-dependent pathways. The maximal absolute increase in FE_{Pi} was less in TPTX rats than in rats with intact parathyroid glands, most likely due to enhanced phosphate reabsorption by the proximal straight tubule in the hypoparathyroid state (43, 44). Further evidence that the phosphaturic effect of sFRP-4 is not PTH-dependent comes from the lack of stimulation of urinary cyclic AMP following sFRP-4 infusion in intact rats. The effect of sFRP-4 observed in OK cells maintained in culture further suggests that sFRP-4 has direct actions on Pi transport.

Infusion of sFRP-4 produces the same defect in vitamin D metabolism observed in TIO. Despite the induction of hypophosphatemia following sFRP-4 infusion, serum 1α, 25-dihydroxyvitamin D concentrations and renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 messenger RNA amounts fail to increase in the predicted physiological manner (Table 1) (45-50). In contrast, phosphate deprivation in normal rats that was associated with mild hypophosphatemia resulted in a significant (3.7-fold) increase in

renal 25-hydroxyvitamin D 1α-hydroxylase cytochrome P450 messenger RNA concentrations. This failure of the vitamin D-endocrine system to respond to hypophosphatemic stimuli is characteristic of TIO and the phenotypically related XLH and ADHR (3-5, 51).

We show that sFRP-4 circulates in the serum of normal humans, suggesting that it could potentially function as a "phosphatonin". sFRP-4 was also detectable in the serum of a patient with TIO, who had an un-resectable tumor and was on treatment with phosphate supplementation and 1α, 25-dihydroxyvitamin D_3. The sFRP-4 concentrations were not elevated relative to the control concentrations. It is possible that treatment of the patient with phosphate and 1α, 25-dihydroxyvitamin D_3 could have suppressed sFRP-4 concentrations. A more comprehensive study of other patients with TIO will be needed prior to making a conclusive statement regarding the usefulness of sFRP-4 concentrations in the diagnosis of TIO. It is of interest that not all patients with TIO have elevations of FGF23 and in some instances cure of the syndrome occurs without changes in FGF23 concentrations (20).

Sodium-dependent phosphate co-transporter mRNA levels were unchanged following infusion of sFRP-4 in rats. This suggests that transcriptional regulation of sodium-phosphate co-transporter mRNA concentrations by sFRP-4 is not critical in modulating phosphate transport. Others have shown that parathyroid hormone does not alter sodium-phosphate co-transporter mRNA concentrations in the kidney but causes a re-distribution in the sodium-phosphate co-transporter protein from the luminal membrane of the

proximal tubular cell into the lysosomal compartment (52-54). Preliminary data suggests that sFRP-4 also causes a re-distribution of sodium-dependent phosphate co-transporter IIa protein in OK cells (data not shown). Further experiments will be needed to precisely define the effects of sFRP-4 on sodium-dependent phosphate co-transporter IIa protein cell biology.

Our data point to a novel function of sFRP-4 in the kidney. Secreted FRP-4 belongs to a family of secreted proteins that contain a cysteine-rich domain that is homologous to the extracellular domain of the Wnt receptors, the Frizzled proteins (55-59). Secreted FRPs modulate the activities of Wnts (60-68). The Wnt signaling pathway plays an important role in renal, bone and cardiac development (69-77). Binding of Wnts to frizzled receptors leads to activation of signals through three different pathways resulting in both transcription and non-transcriptional changes (74-78). sFRP-4 mRNA is detectable in the kidney (58, 79, 80) and these data are supported by our observations that sFRP-4 protein is also detectable in homogenates of rat kidney. We show that sFRP-4 antagonizes Wnt-signaling in the kidney. The binding of Wnts to frizzled receptors normally stabilizes intra-cellular β-catenin by preventing degradation, and decreases the phosphorylation of β-catenin (58, 81-83). Increased phosphorylation of β-catenin suggests that sFRP-4 antagonizes this pathway.

Our findings add to the list of functions attributed to sFRP-4. Secreted FRP-4 expression has been correlated with the presence of apoptosis in several tissues such as osteoarthritic cartilage, but co-localization of sFRP-4 in apoptotic cells and direct induction of

apoptosis by sFRP-4 has not been demonstrated (80, 84, 85). It is also noteworthy that elevated sFRP-4 expression is correlated with several tumor types and proliferative tissues (86, 87). Based on these observations and the diverse expression pattern of sFRP-4, it is likely that additional functions will be identified (88).

In conclusion, we have shown that a protein that is over expressed in tumors associated with renal phosphate wasting and osteomalacia is capable of specifically inhibiting sodium-dependent phosphate transport *in vitro* and selectively increasing the fractional excretion of phosphorus in a parathyroid hormone independent manner *in vivo*. We also show that there is a decrease in serum phosphate concentrations in rats administered this protein for a period of 4 to 8 hours, and that the vitamin D endocrine system fails to respond to hypophosphatemic stimuli following sFRP-4 infusion. Secreted FRP-4 could potentially function as a "phosphatonin," and it is apparent from these studies that there are at least two phosphaturic proteins (FGF23 and sFRP-4) produced by tumors associated with renal phosphate wasting and osteomalacia.

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Figure Legends:

Figure 1: Effect of sFRP-4 on sodium-dependent phosphate uptake in opossum kidney cells maintained in culture. * $p < 0.05$.

Figure 2: Effect of the infusion of sFRP-4 on solute excretion in intact rats. Intact rats were administered sFRP-4 (■) at a dose of 0.3 μg/kg/hr (Group 2) or vehicle (❑) (Group 1) by intravenous infusion over a period of two hours. C1 = equilibration period prior to the infusion of sFRP-4 or vehicle. C2 = experimental period during which sFRP-4 or vehicle was infused. Fractional excretion of inorganic phosphate, sodium and calcium were measured as described in the text. * $p < 0.05$.

Figure 3: Effect of the infusion of sFRP-4 on solute excretion in thyro-parathyroidectomized (TPTX) rats. TPTX rats were administered sFRP-4 (■) at a dose of 0.3 μg/kg/hr (Group 4) or vehicle (❑)(Group 3) by intravenous infusion over a period of two hours. C1 = collection period prior to the infusion of sFRP-4 or vehicle. C2 = collection period during which sFRP-4 or vehicle was infused. Fractional excretion of inorganic phosphate, sodium and calcium were measured as described in the text. * $p < 0.05$.

Figure 4: The effect infusion of vehicle or sFRP-4 (0.3 μg/Kg/hr) for eight hours, or the effect of phosphate deprivation induced by a low phosphate diet phosphate (LPD)

compared with a normal phosphate diet (NPD), on the concentration of 25-hydroxyvitamin D 1α-hydroxylase cytochrome P-40 mRNA concentrations in kidney (* $p<0.05$).

Figure 5: Western blots of renal homogenates obtained from rats infused with vehicle (control) or sFRP-4 for 8 hours. Antibodies against β-catenin (upper panel) or phosphorylated β-catenin (lower panel) were used to detect proteins as described in the Methods section.

Table 1. Effect of Long-Term (8h) Infusion of sFRP-4 on Renal Function in Intact Rats.

	Vehicle (n=5)			sFRP-4 (n=7)		
	C1	C2	C3	C1	C2	C3
GFR (mL/min) ± SE	3.7 ± 0.6	3.2 ± 0.4	2.6 ± 0.6	3.5 ± 0.2	2.8 ± 0.3	2.4 ± 0.5
FE_{Pi} (%) ± SE	7.4 ± 2.2	6.8 ± 1.7	9.9 ± 0.4	8.5 ± 2.6	20.2* ± 3.7	18.2 ± 4.3
FE_{Na} (%) ± SE	1.6 ± 0.4	1.3 ± 0.2	0.8 ± 0.1	1.9 ±0.6	1.8 ± 0.2	2.2 ± 1.1
FE_{Ca} (%) ± SE	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.7 ± 0.3
P_{Pi} (mM) ± SE	2.02 ± 0.15	2.02 ± 0.22	1.83 0.18	1.95 ± 0.10	1.51* ± 0.11	1.53* ± 0.09
P_{Ca} (mg/dl) ± SE	8.0 ± 0.3	9.1 ± 0.3	8.8 ± 0.6	8.7 ± 0.7	9.1 ± 0.3	9.7 ± 0.5
MAP (mm Hg) ± SE	119 ± 6	113 ± 8	108 ± 5	124 ± 10	118 ± 7	120 ± 6
Serum 1α, $25(OH)_2D$ (pg/ml)	62.4 ± 29.2			88.9 ± 26.8		
Renal 25(OH)D 1α-hydroxylase cytochrome P450 mRNA (moles/pg)	$1.41x10^{-13} \pm 3.87x10^{-14}$			$1.37x10^{-13} \pm 1.77x10^{-14}$		
Renal 25(OH) D 24-hydroxylase cytochrome P450 mRNA (moles/pg)	$1.53x10^{-14} \pm 1.38x10^{-15}$			$0.92 x10^{-14} \pm 2.48x10^{-15}$		

GFR, glomerular filtration rate; FE_{Pi}, fractional excretion of phosphate; FE_{Na}, fractional excretion of sodium; FE_{Ca}, fractional excretion of calcium; P_{Pi}, plasma phosphate concentration, P_{Ca}, plasma calcium concentration; MAP, mean arterial pressure. *Indicates a significant difference, ANOVA, $p<0.05$.

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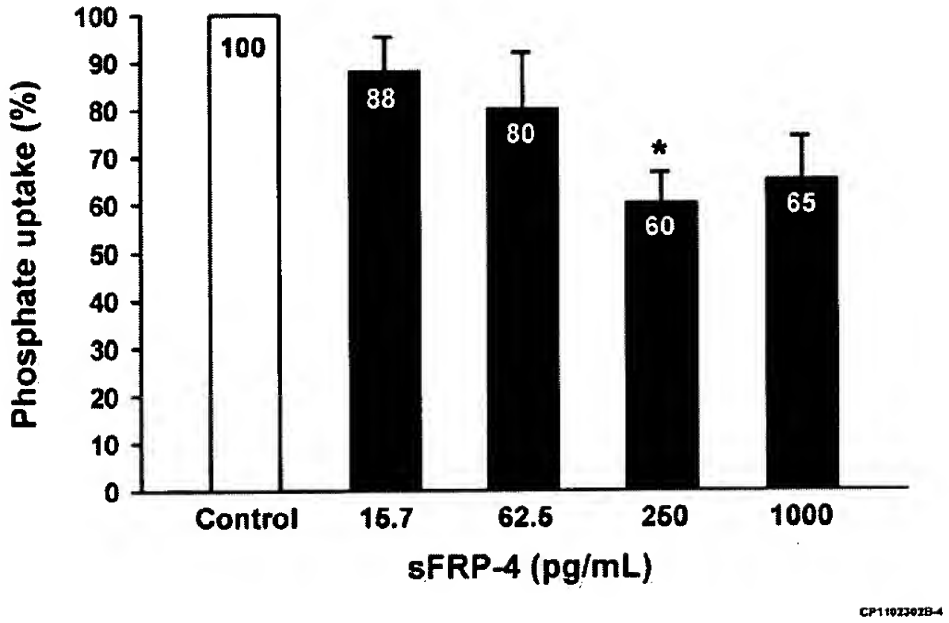
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70
60
50
40
30
20
10
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80
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60
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Control
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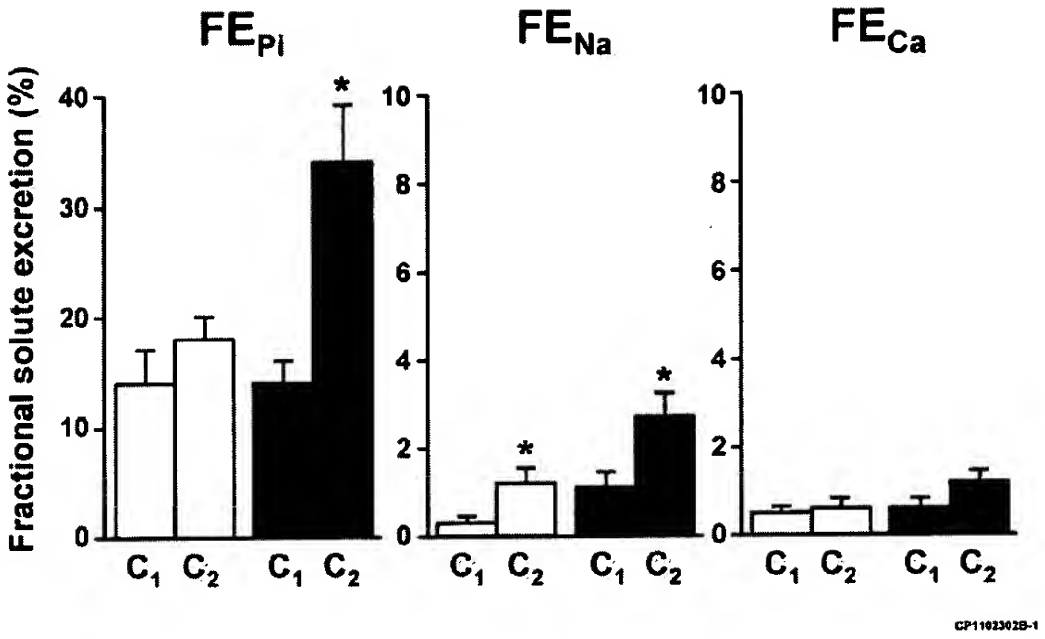
62.5
250
1000
sFRP-4 (pg/mL)

FIGURE 1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FE$_{Ca}$
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8
6
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2
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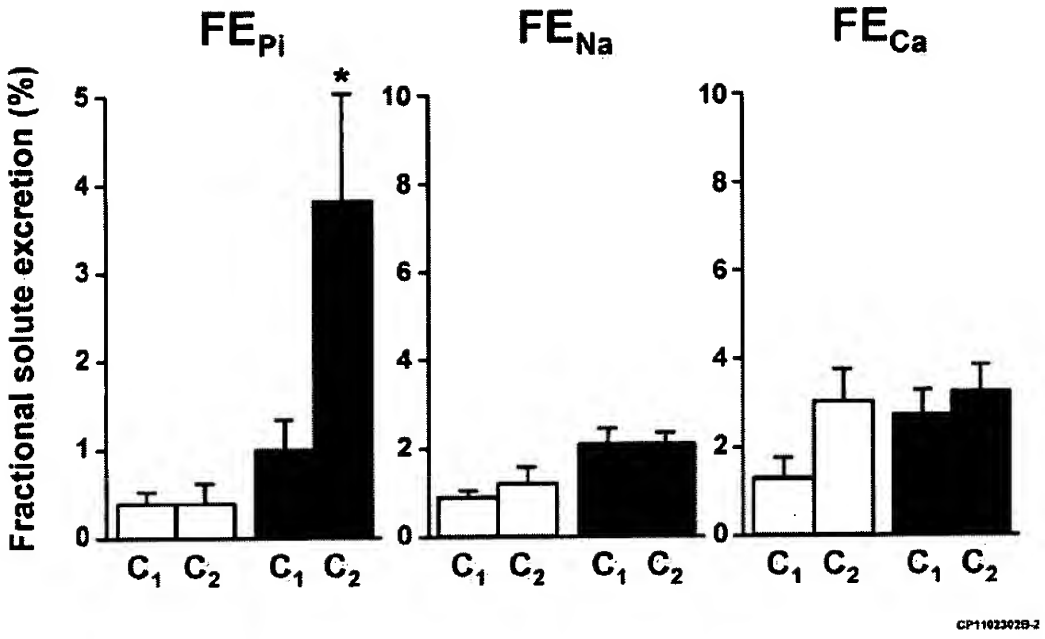
10
8
6
4
2
0
*
*
*
C$_1$ C$_2$ C$_1$ C$_2$
C$_1$ C$_2$ C$_1$ C$_2$
C$_1$ C$_2$ C$_1$ C$_2$
CP1102302B-1

FIGURE 2


FE$_{Pi}$
FE$_{Na}$
FE$_{Ca}$
Fractional solute excretion (%)
*
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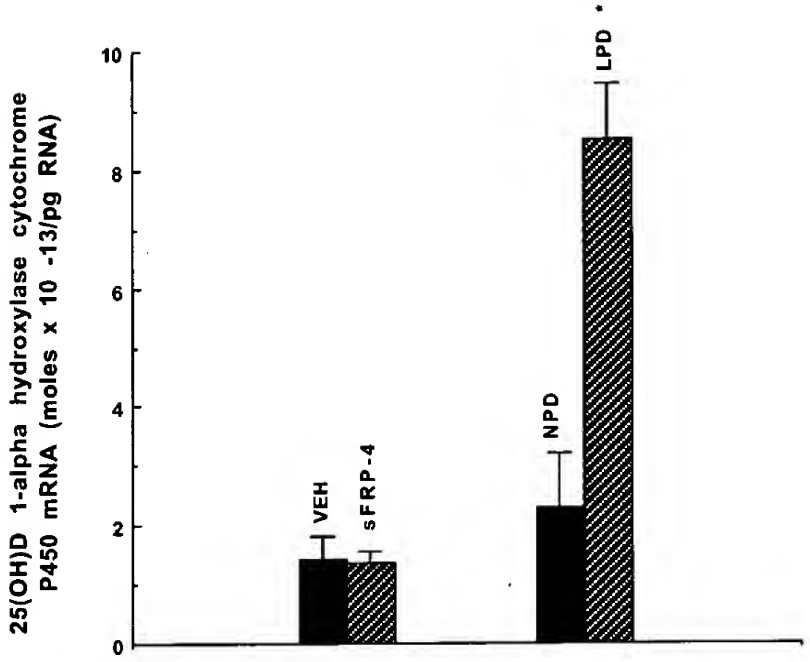
2
3
4
5
0
2
4
6
8
10
0
2
4
6
8
10
C$_1$ C$_2$ C$_1$ C$_2$
C$_1$ C$_2$ C$_1$ C$_2$
C$_1$ C$_2$ C$_1$ C$_2$

FIGURE 3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0
VEH
sFRP-4
NPD
LPD
*

Figure 4

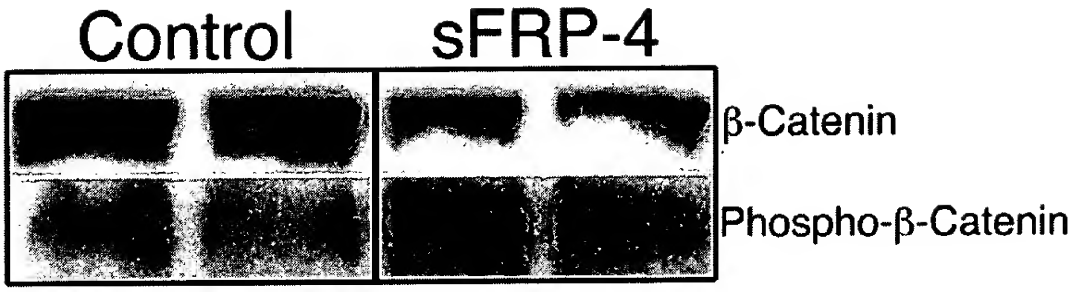

FIGURE 5

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  AUTHORS   Schumann,H., Holtz,J., Zerkow ski,H.R. and Hatzfeld,M.
  TITLE     Expression of secreted frizzl ed related proteins 3 and 4 in human
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  AUTHORS   Abu-Jawdeh,G., Comella,N., Tomita,Y., Brown,L .F., Tognazzi,K.,
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  TITLE     Differential expression of fr pHE: a novel human stromal protein of
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  TITLE     DDC-4, an apoptosis -associated gene, is a secreted frizzled
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  TITLE     A family of secreted proteins  contains homology to the
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the SFRP family that contains a cysteine-rich domain homologous to the putative Wnt -binding site of Frizzled proteins. SFRPs act as soluble modulators of Wnt sig naling. The expression of SFRP4 in ventricular myocardium correl ates with apoptosis related gene expression.

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1: NM_016687. Mus musculus secr...[gi:7710093]

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  AUTHORS   Chang,J.T., Esumi,N., Moore,K., Li,Y., Zhang,S., Chew,C.,
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  TITLE     Cloning and characterization of a secreted frizzled-related protein
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  JOURNAL   Hum. Mol. Genet. (1999)
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  TITLE     A family of secreted proteins contains homology to the
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                     the related pfam00965 family. It has been suggested that
                     the common function of these modules is binding to
                     metzincins. A subset of this family is known as the C345C
                     domain because it occurs in complement C3, C4 and C5"
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1: NM_053544. Rattus norvegicus...[gi:16758311] Links

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ACCESSION   NM_053544
VERSION     NM_053544.1  GI:16758311
KEYWORDS    .
SOURCE      Rattus norvegicus (Norway rat)
  ORGANISM  Rattus norvegicus
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REFERENCE   1  (bases 1 to 1910)
  AUTHORS   Fujita,M., Ogawa,S., Fukuoka,H., Tsukui,T., Nemoto,N., Tsutsumi,O.,
            Ouchi,Y. and Inoue,S.
  TITLE     Differential expression of secreted frizzled-related protein 4 in
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  JOURNAL   J. Mol. Endocrinol. 28 (3), 213-223 (2002)
  MEDLINE   22058279
   PUBMED   12063187
  REMARK    GeneRIF: Differential expression of secreted frizzled-related
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REFERENCE   2  (bases 1 to 1910)
  AUTHORS   Yam,J.W., Chan,K.W., Wong,V.K. and Hsiao,W.L.
  TITLE     Transcriptional activity of the promoter region of rat
            frizzled-related protein gene
  JOURNAL   Biochem. Biophys. Res. Commun. 286 (1), 94-100 (2001)
  MEDLINE   21378144
   PUBMED   11485313
REFERENCE   3  (bases 1 to 1910)
  AUTHORS   Wolf,V., Artuso,L., Dharmarajan,A., Guo,K., Bielke,W. and
            Friis,R.R.
  TITLE     A frizzled related Gene is upregulated in Physiological Apoptosis
  JOURNAL   Unpublished
REFERENCE   4  (bases 1 to 1910)
  AUTHORS   Guo,K., Wolf,V., Dharmarajan,A., Feng,Z., Bielke,W., Susanne,S. and
            Friis,R.R.
  TITLE     Apoptosis-associated Gene Expression in Corpus luteum of the Rat
  JOURNAL   Unpublished
COMMENT     PROVISIONAL REFSEQ: This record has not yet been subject to final
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                     the related pfam00965 family. It has been suggested that
                     the common function of these modules is binding to
                     metzincins. A subset of this family is known as the C345C
                     domain because it occurs in complement C3, C4 and C5"
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